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The radioresistance biological function of the SCF/kit signaling pathway is mediated by the zinc-finger transcription factor Slug

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Radiation-induced destruction of the hematopoietic system is the primary cause of death based on the findings that transfer of normal bone marrow cells prevents death from lethal irradiation. The stem cell factor-c-kit signaling pathway (SCF/c-kit) has been previously implicated in the hematopoietic recovery which prevents death from lethal irradiation, but the molecular mechanisms that mediate this biological effect are unknown. Since mutations on *SCF*, *c-kit* and *Slug* genes have a similar phenotype in mice, we examined if Slug could complement the radiosensitivity of kit-deficient mice. In this report, we show that Slug acts as a radioprotection agent as lack of Slug results in increased radiosensitivity. This effect cannot be recovered by activating SCF/c-kit in lethally irradiated Slug-deficient mice, as SCF-treated mice did not demonstrate stimulation of hematopoietic recovery leading to survival of the Slug-deficient mice. We found that we could complement the hematopoietic failure in lethally irradiated c-kit-deficient mice by transducing them with a TAT-Slug protein. We conclude that the zinc-finger transcription factor Slug is absolutely necessary for survival from lethal irradiation and identify Slug as the molecular target that mediates the radioprotection through SCF/c-kit. These results indicate that Slug may be a molecular component conferring radioresistance to cancer cells.
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Introduction

Stem cell factor (SCF) is the product of the steel (Sl) locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor encoded by the white spotting (W) locus of the mouse (Sarvella and Russell, 1956; Russell, 1979; Silvers, 1979; Chabot *et al*., 1988; Geissler *et al*., 1988; Copeland *et al*., 1990; Huang *et al*., 1990; Martin *et al*., 1990; Williams *et al*., 1990; Zsebo *et al*., 1990). The c-kit ligand appears to have unique and nonredundant activities on primitive progenitor cells (Witte, 1990). In this regard, there is ample evidence that SCF, in the absence of other cytokines, selectively promotes viability rather than proliferation of primitive murine hematopoietic progenitor cells (Fleming *et al*., 1993). However, the molecular mechanisms that provide biological specificity to the SCF/c-kit signaling pathway in the formation and migration of c-kit$^+$ cells are largely unknown. Recently, we have shown that Slug-mutant mice have phenotypic characteristics similar to Sl and W mutant mice and identified that the zinc-finger transcription factor Slug is a molecular target that contributes to the biological specificity of the SCF/c-kit signaling pathway (Perez-Losada *et al*., 2002). *Kit* and *Slug* genes coordinately modulate the development and survival of melanocyte and hematopoietic lineages in compound-deficient mice (Perez-Losada *et al*., 2002; Sanchez-Martin *et al*., 2002).

The biological properties of SCF have demonstrated that the action of SCF is on very primitive cell populations. In this regard, endogenous production of SCF is required for survival from lethal irradiation (Neta *et al*., 1993; Zsebo *et al*., 1993). Survival after irradiation with a dose lethal ($LD_{100/30}$, radiation dose lethal to 100% of mice in 30 days) is based on recovery of impaired hematopoietic function as transplantation of normal bone marrow cells into lethally irradiated animals and humans results in long-term survival and provides proof that the hematopoietic system is crucial for defense against the lethal complications induced by radiation. Sl mutant animals have an LD_{100} of 200–300 rad, in contrast to an LD_{100} of their congenic counterparts being in the range of 720—900 rad (Harrison and Russell, 1972; Kaczmarek *et al*., 1988), showing that lack of SCF results in increased radiosensitivity. Since mutations on *SCF*, *c-kit*, and *Slug* genes have a similar phenotype that affects hematopoiesis (Perez-Losada *et al*., 2002), and the radiosensitivity is a characteristic phenotypic abnormality seen in Sl and W mutants *in vivo* (Harrison and Russell, 1972; Kaczmarek *et al*., 1988), we examined if Slug could complement the radiosensitivity of kit-deficient mice. Here we show that lack of Slug results in increased radiosensitivity and this effect cannot be recovered by

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treating lethally irradiated Slug-mutant mice with SCF stimulation of hematopoietic recovery. In contrast, we could complement the hematopoietic failure following DNA damage after γ-irradiation in c-kit-deficient mice by transducing them with a TAT-slugh protein. These results probe that the radioresistance biological function of SCF is mediated by the zinc-finger transcription factor Slug.

Results

Radioprotective potential of Slug-deficient cells in response to γ-irradiation

Slug-deficient bone marrow cells were analysed functionally *in vivo* after DNA damage induced by γ-irradiation. Initially, Slug $+/+$, $+/-$ and $-/-$ mice were irradiated at various doses ranging from 100 to 1200 rad (1 rad = 0.01 Gy) to determine an LD of irradiation for the Slug mice used in these studies. A total of 50% of mice survived for 30 days given an irradiation dose of 275 rad ($LD_{50/30}$). Similarly, the $LD_{90/30}$ was obtained at 300 rad, and the $LD_{100/30}$ was obtained at 350 rad (Figure 1). Thus, Slug $-/-$ mice had an $LD_{100/30}$ of 350 rad in contrast to an $LD_{100/30}$ of their Slug $+/+$ congenic counterparts being in the range of 1100–1150 rad. Subsequent studies were therefore performed at 350 rad to obtain a lethal dose of irradiation for Slug-deficient mice.

As the heterozygous loss-of-function of Slug leads to phenotypic abnormalities (Perez-Losada *et al*., 2002; Sanchez-Martin *et al*., 2002), we also determine the LD of irradiation for the Slug $+/-$ mice. The LD100/30 of Slug $+/-$ mice was obtained at 1100 rad (Figure 1), being similar to the $LD_{100/30}$ of their Slug $+/+$ congenic counterparts. These results indicate that a loss-of-function mutation of Slug in one allele does not result in increased radiosensitivity.

To determine if hematopoietic recovery was crucial in the recovery from these doses of irradiation, Slug $-/-$ and $+/-$ mice irradiated at 350 and 1100 rad, respectively, were transplanted with bone marrow cells from syngeneic normal donors. At 4 h postirradiation, i.v. injection of bone marrow resulted in 100% survival of mice at both doses of irradiation compared to 0% survival of control animals that did not receive a bone marrow transplant (BMT). The serum chemistry data showed no significant differences, suggesting that there were no major organ failures that could account for the differences in survival in the groups. Blood cultures of peripheral blood detected the presence of gut-related bacteria in 10 of 10 mice which did not recieve a BMT compared to none of nine BMT-treated mice.

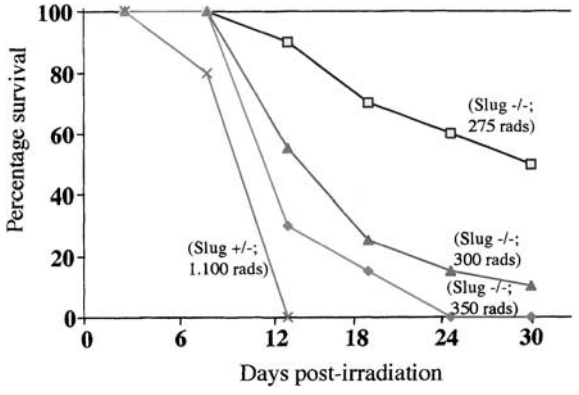

Figure 1 Effect of irradiation on survival of Slug $-/-$ mice. Slug $-/-$ and $+/-$ mice were irradiated at various doses to determine a lethal dose of irradiation. The survival of Slug $+/-$ and wild-type (wt) mice was not influenced by irradiation at low doses. The radiation dose was given as a split dose of equal intensity, 4 h apart

Normal p53 activation in Slug-deficient bone marrow (BM) cells in response to DNA damage

We have found that Slug-deficient BM cells are radiosensitive to DNA damage induced by γ-irradiation. p53 is centrally involved in the cellular response to radiation (Lee *et al*., 1994). Exposure to ionizing radiation causes an increase in the intracellular levels of p53 (Kastan *et al*., 1991). Thus, we next explored if the radioprotective potential of Slug was based on interference with p53 activation. As shown in Figure 2, we measured the p53 protein levels at different time points in BM cells derived from both Slug $-/-$ and Slug $+/+$ mice after DNA damage induced by γ-irradiation. The activation of p53 in both control and Slug-deficient cells was similar (Figure 2), indicating that p53 regulation in response to DNA damage is not affected in Slug-deficient cells. These results are in agreement with previous data (Inoue *et al*., 2002) and suggest that Slug does not require p53 for its radioprotective function.

Role of SCF on hematopoietic recovery in lethally irradiated Slug-deficient mice

Radioprotective potential was found only in wild-type BM whereas Slug-deficient BM did not protect from lethal irradiation. The radioprotective potential in mice is subject to modulation by SCF/c-kit interactions (reviewed by Broudy, 1997). Treatment of irradiated mice with SCF results in increased survival compared to irradiated controls (Neta *et al*., 1993; Zsebo *et al*., 1993). Since SCF radioprotection is manifest both after i.p. and i.v. routes of injection for rSCF in mice (Zsebo *et al*.,

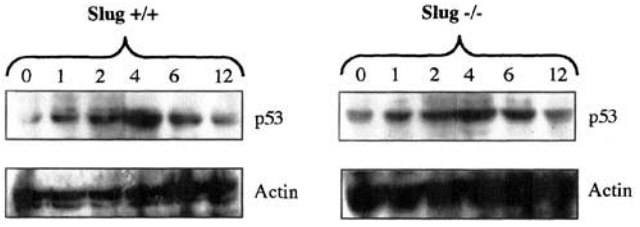

Figure 2 Levels of p53 protein in BM cells after γ-irradiation. p53 protein was detected by Western blotting in Slug $+/+$ and Slug $-/-$ BM cells. Actin was used as a loading control. The time points are in hours

1993), the i.v. route was used in our experiments. The combination of pre and post-treatment with SCF (−20, −2, and +4 h) results in 100% survival of Slug +/+ and +/− mice irradiated at 1100 rad (Table 1).

To determine the extent to which SCF could protect Slug −/− mice against otherwise lethal irradiation, Slug −/− mice were irradiated at 350 rad and injected with SCF or carrier. By day 30, 0% of Slug −/− animals survived. In comparison, none of the SCF-treated Slug +/+ and +/− animals died postirradiation, and the animals continued to survive past the 30-day study period. The BM cellularity, including CFU-GM, CFU-E, and BFU-E, never recovered in Slug −/− SCF-treated mice (Figure 3). By day 3 postirradiation, the BM cellularity dropped to ~4% of preirradiation levels (Figure 3), whereas Slug +/− SCF-treated mice had a dramatic increase in cellularity by day 8 and had almost returned to preirradiation levels by day 21 (Figure 3). These results were concordant with peripheral blood hematology which showed no significant differences between SCF-treated and untreated Slug −/− mice (Perez-Losada *et al*., 2002) and we did not observe the expected increase in WBCs, RBCs, and platelet values during the 2–3 weeks postirradiation.

Table 1 Effect of SCF on survival of $LD_{100/30}$ irradiated mice

Genotype	*Treatment*	*Dead/total*	*% survival*
Slug−/−	Excipient	40/40	0
Slug−/−	SCF	40/40	0
Slug+/−	Excipient	40/40	0
Slug+/−	SCF	0/40	100
Slug+/+	Excipient	40/40	0
Slug+/+	SCF	0/40	100

The excipient or the SCF was injected i.v. at 20 and 2 h prior to irradiation and 4 h after the last dose of irradiation. Slug −/− were irradiated at 350 rad and Slug +/− and +/+ at 1.100 rad

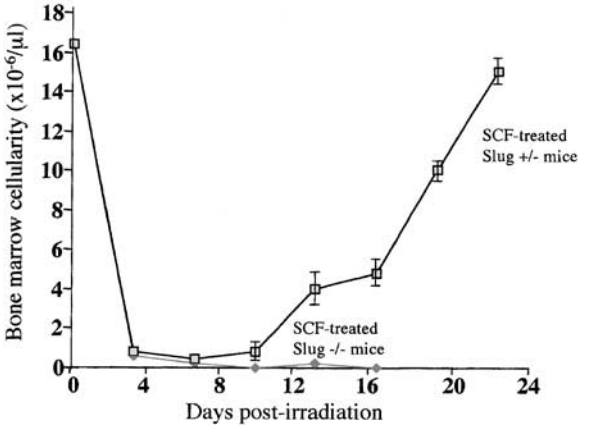

Figure 3 Bone marrow cellularity of mice irradiated at $LD_{100/30}$ and treated with SCF. The SCF was injected i.v. at 20 and 2 h prior to irradiation and 4 h after the last dose of irradiation. The BM cellularity of wt mice was similar to Slug +/− mice irradiated at $LD_{100/30}$ and treated with SCF. Data presented are the mean ± s.e.m. for four mice at each time point

Rescue of aplasia induced by lethal irradiation in kit*-mutant mice by TAT-Slug*

Slug is a downstream molecular target of the SCF/c-kit signaling pathway (Perez-Losada *et al*., 2002), thus we next explored if Slug could defend against the hematopoietic failure induced by radiation in *kit*-mutant mice. As there were no pharmacological mechanisms to activate Slug, we turned to the transduction of Slug: TAT-Slug (Figure 4a). An amino-terminal TAT-Slug protein was generated (Nagahara *et al*., 1998). Immunoblot and microscopic analysis revealed that the TAT-Slug fusion protein was rapidly transduced into cultured cells reaching near maximum intracellular concentrations in less than 20 min (Figure 4b–d).

We next intraperitoneally (i.p.) injected TAT-Slug protein into Slug −/− mice (Schwarze *et al*., 1999). Immunoblot analysis of BM cells isolated 120 min after i.p. injection demonstrated the presence of TAT-Slug protein reaching maximum concentrations after injection of 250 μg of TAT-Slug fusion protein (Figure 4e). To test the effect of TAT-Slug in lethally irradiated Slug-deficient mice, we administrated a single dose of 250 μg of TAT-Slug protein immediately after lethal irradiation. All 10 Slug-deficient mice survived after treatment with the TAT-Slug protein compared with none of 10 Slug-deficient mice treated with TAT-control protein (Figure 4f). These results demonstrate that the administration of TAT-Slug can completely complement the recovery from aplasia induced by lethal irradiation in Slug-deficient mice.

We next test the effect of TAT-Slug in irradiated kit-deficient mice by administration of a single dose of 250 μg of the TAT-Slug protein immediately after lethal irradiation. All 10 kit-deficient mice survived after tretament with the TAT-Slugh protein compared with none of 10 kit-deficient mice treated with TAT-control protein (Figure 4f). The TAT-Slugh protein protected kit-deficient BM cells from apoptosis after lethal irradiation (Figure 5a). The bone marrow cellularity of kit-deficient TAT-Slug-treated mice recovered and almost returned to preirradiation levels and these results were concordant with peripheral blood hematology (Figure 5B). Thus, TAT-Slug appears to protect kit mutant mice from lethal irradiation, indicating that Slug mediates the radioprotective potential of the kit/SCF signaling pathway.

Discussion

The radioprotective function of the SCF/c-kit signaling pathway is mediated by Slug

The biological events controlled by the SCF/c-kit signaling pathway are implicated in the generation and migration of hematopoietic stem cells (Witte, 1990; Fleming, 1993). In this study, we have investigated if the survival from lethal irradiation governed by the SCF/c-kit signaling pathway is mediated by the Slug transcription factor. Accordingly, as the absence of Slug impairs

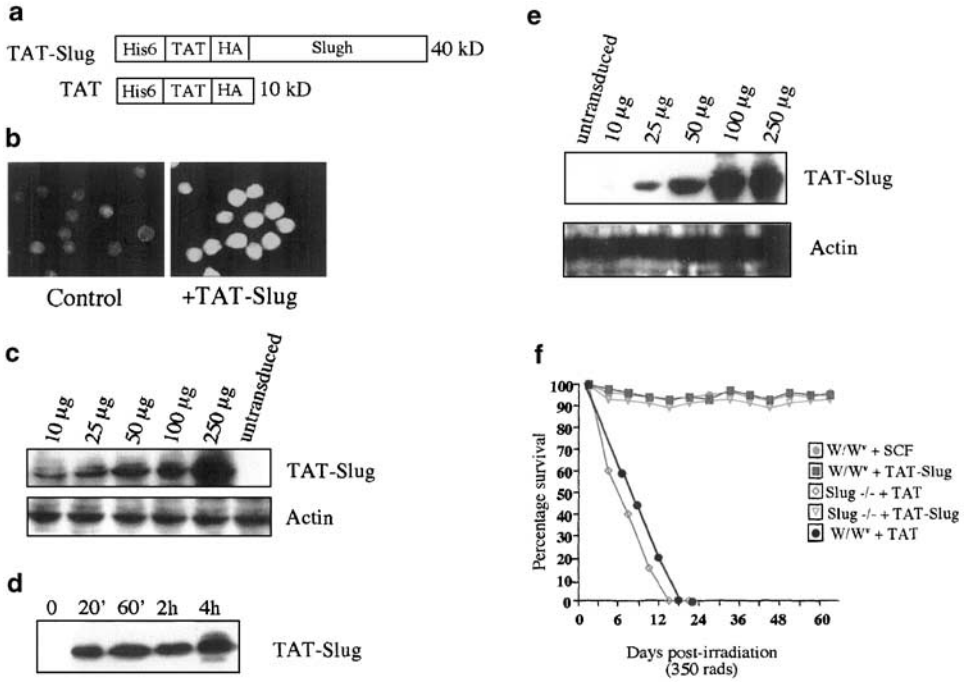

Figure 4 Transduction of TAT-Slugh into mice. (**a**) Diagram of TAT-Slug fusion. (**b**) Immunofluorescence of untransduced Ba/F3 cells (left panel) and Ba/F3 cells transduced with TAT-Slug protein. TAT-Slug protein was detected with the 12CA5 monoclonal antibody, which detects the HA epitope. (**c**) Immunoblot of Ba/F3 cells 60 min after being transduced with different amounts of TAT-Slug protein. (**d**) Immunoblot of Ba/F3 cells transduced with 50 μg of TAT-Slug protein at different time points. (**e**) Immunoblot of BM cells isolated from Slug $-/-$ mice 120 min after i.p. injection of different amounts of TAT-Slug fusion protein. (**f**) Effect of irradiation on survival of *kit*-deficient mice transduced with TAT-Slug fusion protein. Mice were irradiated at 350 rad given as a split dose. SCF was injected i.v. at 20 and 2 h prior to irradiation and 4 h after the last dose of irradiation and TAT proteins were injected i.p. with a single dose of 250 μg immediately after irradiation

the development of hematopoietic stem cells (Perez-Losada *et al*., 2002) and mutations at either W or Sl, and *Kit* and *Slug* genes coordinately modulate the development and survival of melanocyte and hematopoietic lineages in compound-deficient mice (Perez-Losada *et al*., 2002; Sanchez-Martin *et al*., 2002), we first analysed if the absence of Slug results in increased radiosensitivity. The data presented in this study clearly demonstrate that mice mutated at the Slug locus are radiosensitive. These results corroborate that Slug is absolutely necessary for survival from lethal irradiation of unmanipulated mice (Inoue *et al*., 2002). As Slug-mutant mice have a normal SCF/c-kit signaling pathway (Perez-Losada *et al*., 2002), we further demonstrate that the activation of c-kit by SCF specifically cannot recover this effect in Slug-mutant mice. In contrast, we could complement the hematopoietic failure following DNA damage after γ-irradiation in c-kit-deficient mice by transducing them with a TAT-Slug protein, showing that the SCF stimulation of hematopoietic recovery after lethal irradiation is mediated by Slug.

These findings are congruent with our proposed model in which stem cells harboring the c-kit receptor would express Slug, promoting survival of the cell, with dependence of the required external signal (SCF) and allowing cells to migrate outside their normal environment. If this is not achieved in a specific period of time, they would undergo apoptosis as they have been deprived of required external signals to keep Slug expression (Perez-Losada *et al*., 2002).

Importance of the Slug-dependent radioresistance in cancer therapy

Our results show that the radioresistance confers to HSC cells by SCF/kit signaling pathway is mediated by Slug. The c-kit receptor is involved in both leukemias and solid tumors. Mutations resulting in constitutive activation of c-kit have been described in acute myeloid leukemias (Furitsu *et al*., 1993; Nagata *et al*., 1995), small cell lung cancer (Matsuda *et al*., 1993), gynecological tumors (Inoue *et al*., 1994), breast carcinomas (Hines *et al*., 1995) and colonic tumors derived from intersticial cells of Cajal (a cell type that is SCF dependent) (Hirota *et al*., 1998; Nishida *et al*., 1998). Thus, constitutive activation of c-kit could confer radioresistance properties to the tumor cells. Moreover, recent findings show that Slug is also expressed in t(17;19) leukemic cells (Inukai *et al*., 1999), in rhabdomyosarcoma cells expressing the translocation PAX3-FKHR (Khan *et al*., 1999), and in cells expressing BCR-ABL (JPL *et al*., unpublished observations). Thus, Slug may be a common component providing radioresistance to tumor cells. As such, Slug might therefore constitute an attractive target in the treatment of human cancer.

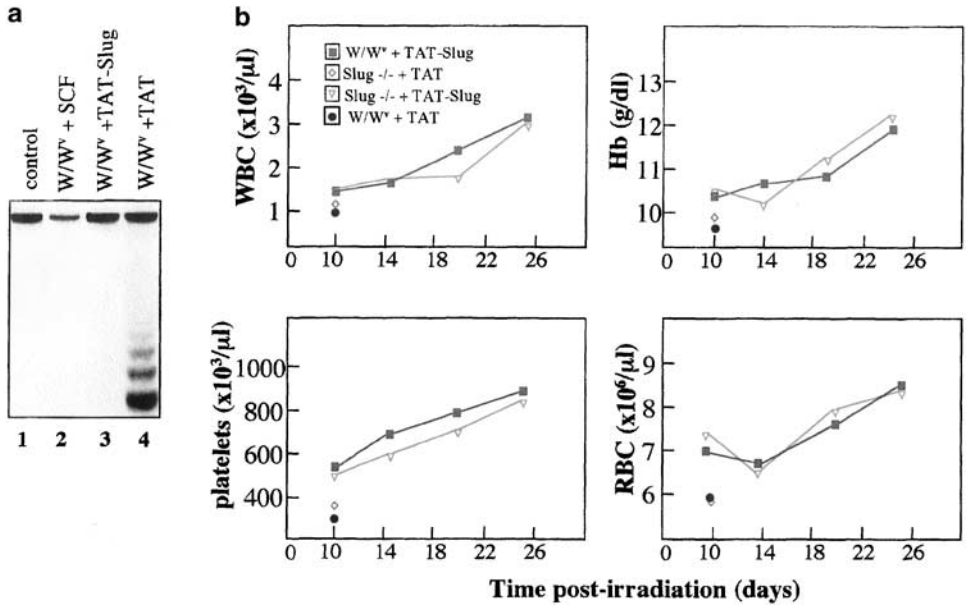

Figure 5 Rescue of aplasia induced by lethal irradiation in *kit*-mutant mice by TAT-Slug. (**a**) Effect of SLUG on the survival of W/W^v BM cells after irradiation. Cell death is accompanied by nucleosome laddering after irradiation. Low molecular weight DNA was isolated 6 h after irradiation with 350 rad from control mice (lane 1), W/W^v mice treated with SCF – the SCF was injected i.v. at 20 and 2 h prior to irradiation and 4 h after the last dose of irradiation – lane 2, WAV^v mice injected i.p. with a single dose of 250 μg of TAT-Slug fusion protein immediately after irradiation (lane 3), and W/W^v mice injected i.p. with a single dose of 250 μg of TAT control protein immediately after irradiation (lane 4). DNA was end-labeled, resolved by electrophoresis in a 2% agarose gel, and visualized by autoradiography. (**b**) Peripheral blood hematology of mice irradiated at 350 rad and treated with TAT-Slug fusion protein or TAT protein. Similar results were obtained after studying five mice per group. WBC count, platelets, RBC count, and haemoglobin (Hb)

In this sense, Slug has been shown to play similar roles to Snail in several systems (Cheng *et al*., 2001; Blanco *et al*., 2002; Del Barrio and Nieto, 2002; Hajra *et al*., 2002), and, thus, other members of the Snail family of transcription factors could also be involved in similar biological functions to those described herein to Slug.

Methods

Mice

Mice heterozygous and homozygotes for the Slugh$^{\Delta 1}$ mutation generated by removing the genomic sequences of the entire Slugh protein-coding region (Slugh$^{\Delta 1}$ mutant mice) have been previosly described (Jiang *et al*., 1998). W/W^v mice and breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All the procedures were approved by the Institutional Animal Care Committee. Mice were irradiated at various doses using a cesium source, given as a split dose of equal intensity, 4 h apart. Mice were injected i.v. in the tail vein SCF (Sigma) at a dose of 100 μg/kg or saline containing 0.1% fetal bovine serum (excipient for SCF dilutions) at various time intervals. All recipients were maintained in microisolator cages on sterilized food and acidified sterile water. The number of surviving mice was recorded daily for 30 days.

Bone marrow analysis

Bone marrow cells ($0.25–1.0 \times 10^5$ cells/plate) isolated from normal and Slug-mutant mice were seeded into FBS-free semisolid culture plates (Stem Cell Technologies). Colony growth was stimulated with the following combinations of recombinant growth factors: rat stem cell factor (100 ng ml; Sigma), mouse interleukin 3 (10 ng ml; Sigma), and human EPO (2 U/ml; Roche) for burst-forming unit erythroid (BFU-E) growth. The growth of colony-forming unit-erythroid (CFU-E)-derived colonies was stimulated with EPO alone (2 U/ml). The growth of myeloid colonies (CFU-GM) was stimulated with recombinant murine GM-CSF (10 ng ml; SIGMA) in the presence or absence of SCF (100 ng ml; Sigma). The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2 in air and scored either 3 days (for CFU-E-derived colonies) or 7 days (for GM-CSF and BFU-E-derived colonies) following initiation of the culture. The frequency of the colonies was determined in triplicate cultures.

Peripheral blood analysis

Peripheral blood samples were collected into EDTA-containing tubes by retro-orbital bleedings and were subjected to automated complete blood cell counts. For blood cultures, blood was collected in sterile 1-ml insulin syringes coated with 70 μl of a 3.8% trisodium citrate solution (used as an anticoagulant that does not inhibit aerobic growth). An aliquot (0.1 ml) of blood was inoculated into tubes containing (i) trypticase soy broth or (ii) fluid thioglycolate medium and incubated at 35°C for 7 days, after which time the presence of bacteria was determined using standard techniques.

Serum chemistry

Whole serum was collected without the addition of anticoagulants, and serum chemistry values were calculated using

an automated serum chemistry analyzer (Beckman). Values for blood urea nitrogen, potassium, glucose, albumin, total protein, and creatinine were analysed for 10 animals per group per day.

Transduction of TAT-Slug into mice

Slug −/− and kit (W/W^v) mutant mice (8-week-old) were injected intraperitoneally with 250 μg of either TAT-Slug fusion protein or TAT protein in 500 μl of phosphate-buffered saline (PBS). Treated mice were irradiated and analysed as previously described.

Genetic TAT-Slug fusion was generated by insertion of the Slug open reading frame DNA into the pTAT-HA plasmid (Nagahara *et al*., 1998) and they were then transferred into BL21(DE3)LysS bacteria. Both TAT and TAT-Slug fusion proteins were purified as described (Nagahara *et al*., 1998) by sonication in 8 M urea followed by passage over an Ni-NTA column (Qiagen), desalted over aPD-10 column (Amersham Pharmacia) into PBS, then flash frozen in 10% glycerol and stored at –80°C.

Labelling of cells

Ba/F3 cells transduced with the TAT-Slug fusion protein were analysed by immunofluorescence labeling as follows. Cells were fixed in 4% (w/v) paraform aldehyde for 15 min, washed in PBS, and permeabilized in methanol for 2 min. After blocking in 5% FCS in PBS for 30 min, the anti-HA monoclonal antibody (12CA5) was used to detect transduced cells. Fluorescent cells were visualized by epifluorescent microscopy. Images were recorded on the confocal scanning microscope.

Immunoblot

Bone marrow cells were collected by flushing the marrow cavity of femurs. Western blot assays were carried out using extracts from 1×10^7 BM cells or Ba/F3 cells per lane. Mouse p53 was detected using the anitbody FL-393 (Santa Cruz), TAT-Slugh fusion protein was detected using the anti-HA antibody (12CA5). The polyclonal antibody C-11 (Santa Cruz) was used to detect actin.

DNA analysis

Low molecular weight DNA was isolated as follows. Cells were collected into 1.5 ml of culture medium and microcentrifuged for 1 min at 1500 r.p.m (400 *g*), and the pellet was suspended in 300 ml of proteinase K buffer. After overnight incubation at 55°C, DNA was ethanol-precipitated, suspended in 200 ml of TE buffer containing 50 mg/ml of RNase A, and incubated at 37°C for 2 h. DNA was extracted with phenol and chloroform and precipitated with ethanol. Aliquots of DNA (2 mg) were end-labeled with a32-dCTP and electrophoresed on 2% agarose gels. After electrophoresis, the gel was blotted onto Hybond-N (Amersham) and autoradiographied for 2 h at −70°C.

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